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RESEARCH ARTICLE

The Difference between the Effect of Green Tea Cream and Tocopherol on Decreasing Level of Tyrosinase Enzyme and Amount of Melanin in Rattus norvegicus Exposed to UVB Rays

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ABSTRACT

Introduction: *Green tea and tocopherol are potent antioxidants used to treat melasma. However, whether green tea or tocopherol is superior remains unclear.* **Objective:** *To compare the effectiveness between green tea and tocopherol in decreasing the amount of melanin and tyrosinase level in wistar rats exposed to UVB rays.*

Methods: *The experimental study took 30 male Wistar rats randomly and divided them into 3 groups. The control group (C-G) was given basic topical cream, GT-G was given green tea topical cream and TC-G was given tocopherol topical cream. All rats were exposed to UVB every Monday, Wednesday and Friday for 4 weeks, whereas topical creams were smeared every day. Topical creams smearing on the same day with UVB exposure was performed 20 minutes before exposure and 4 hours after UVB exposure. The doses of UVB were 50 mJ/cm2 in first week, 70mJ/cm2 in the second week and 80mJ/cm2 in the third and fourth weeks. The amount of melanin was measured using pixel method and the tyrosinase level was measured using ELISA.*

Results: *Anova analysis indicates that the amount of melanin and tyrosinase level are significantly different between groups, p<0.05. Post Hoc LSD analysis indicates that the amount of melanin in GT-G and TC-G are significantly lower than that of C-G, p<0.05. The amount of melanin in GT-G is lower than that of TC-G, p<0.05. The tyrosinase level in GT-G is significantly lower than that of C-G and TC-G, p<0.05. Meanwhile, the tyrosinase level in TC-G is lower than that of C-G but insignificantly, p>0.05.*

Conclusion: *Green tea topical treatment is significantly capable of decreasing the amount of melanin and tyrosinase level better than tocopherol.*

Keywords: *Green tea, tocopherol, amount of melanin, tyrosinase level*

ABSTRAK

Latar Belakang: Teh hijau dan tocoferol merupakan antioksidan kuat yang telah digunakan untuk mengobati melasma. Namun, belum diketahui mana yang lebih baik. **Tujuan:** mengevaluasi perbedaan efek penggunaan teh hijau dengan tokoferol terhadap jumlah melanin dan kadar tirosinase pada tikus yang dipapar Ultraviolet B (UVB).

Metode: Penelitian eksperimental, menggunakan 30 ekor tikus wistar jantan, dibagi menjadi 3 kelompok secara random. Kelompok kontrol (C-G), dioles krim dasar; Kelompok teh hijau (GT-G), dioles krim teh hijau; Kelompok tokoferol (TC-G), dioles krim tokoferol. Paparan sinar UVB dilakukan pada semua tikus tiap Senin, Rabu, dan Jumat selama 4 minggu, sedangkan pengolesan krim dilakukan setiap hari. Pengolesan krim pada hari yang sama dengan paparan UVB, dilakukan 20 menit sebelum dan 4 jam sesudah paparan UVB. Dosis UVB yang diberikan adalah 50 mJ/cm2 pada minggu I, 70 mJ/cm2 minggu II, 80 mJ/cm2 minggu III dan IV. Jumlah melanin diukur dengan metoda pixel dan kadar tirosinase diukur dengan metode ELISA.

Hasil: Analysis ANOVA menggambarkan bahwa jumlah melanin dan kadar tirosinase di antara kelompok, berbeda bermakna, p<0.05. Analisis *Post Hoc* LSD menunjukkan bahwa jumlah melanin pada GT-G dan TC-G lebih rendah bermakna dibanding C-G, p<0.05. Jumlah melanin pada GT-G lebih rendah bermakna dibanding TC-G, p<0.05. Kadar tirosinase pada GT-G lebih rendah bermakna dibanding C-G dan TC-G, p<0.05. Sedangkan kadar tirosinase pada TC-G lebih rendah dibanding C-G, namun tidak bermakna, p>0.05

Kesimpulan: pemberian krim teh hijau mampu menurunkan jumlah melanin dan kadar tirosinase secara bermakna dibanding tokoferol.

Kata kunci: Teh hijau, tokoferol, jumlah melanin, kadar tirosinase

INTRODUCTION

Melasma is a problem to people living in tropical region, especially women. A woman does not feel confident when she has dull face. Melasma, a cause of dull face, drives women to search for any treatment to get rid of it (Hadiyati PU, Sibero HT, & Apriliana E, 2014; Videira, Moura, & Magina, 2013). Melasma incidents to pregnant women of Hispanic race in Mexico are up to 80%. In Indonesia, its incidents are expected to be at a rate of 0.2 - 4% of those with skin

disease(Jusuf, 2017). Many medicines have been used to treat this melasma, which may reduce the intensity of melasma, but with side effects, at high price and at high rate of recurrence(Jusuf, 2017). Therefore, alternative treatments for melasma need to be developed from plant-derived material, such as green tea. Green tea contains flavonoid, particularly epigallocatechin gallate (EGCG), and is a potent antioxidant able to reduce tyrosinase enzyme activity because of UVB exposure (Young Chul Kim, Choi, & Park, 2015)

Moreover, green tea seed contains a number of kaempferol, a flavonoid and potent antioxidant, able to prevent reduction in antioxidant resulting from enzyme activity because of UVB rays exposure (Prasanth, Sivamaruthi, Chaiyasut, & Tencomnao, 2019). In addition, polyphenol in green tea is reported to reduce tyrosinase enzyme activity, skin damage and skin wrinkling resulting from UVB radiation (Lim et al., 2014). Besides green tea, tocopherol may also be used to reduce melisma (Keen & Hassan, 2016). McVean et al. report that single application of tocopherol cream 5% may be highly accumulated in cell and cell nucleus, protecting from damage to DNA resulting from UVB rays (Nichols & Katiyar, 2011). Application of topical tocopherol acetate 5% to lab rat radiated with UV rays reduces malondialdehyde (MDA) level. MDA is a product of lipid peroxidation degradation, and is the marker of skin aging process. Referring to the researches, both green tea and tocopherol are deemed to potentially reduce melisma or skin hyperpigmentation (Ayala, Muñoz, & Argüelles, 2014) however, which one is superior to the other is still unclear.

Tocopherol contains 8 types of compounds consisting of alpha-, beta-, gamma-, delta-tocopherols and alpha-, beta-, gamma-, delta-tocotrienols. The most abundant tocopherol compound found in diet is gamma tocopherol, while alpha tocopherol is the most vitamin E existing in human tissue and importantly serving as antioxidant (Keen & Hassan, 2016). In cell membrane, tocopherol may prevent lipid oxidation, particularly Poly Unsaturated Fatty Acid (PUFA).In mitochondria, tocopherol protects metabolic pathway which will transform energy fuel into ATP. In fat tissue, antioxidant from tocopherol attacks lipid peroxide as the result of lipid and free radicals reaction (Suárez-Jiménez et al., 2016). The concentration of gamma tocopherol in skin is higher than that of alpha tocopherol. Gamma tocopherol serves to prevent sunburn and oxidative stress on skin resulting from UVB exposure (Keen & Hassan, 2016). Single tocopherol is able to reduce the intensity of minimal melisma caused by inhibition of lipid peroxidation of melanocyte membrane, increase cell glutathione level, and inhibit tyrosinase enzyme (Keen & Hassan, 2016).

Green tea contains chemical compounds consisting of four groups, namely non-phenol, phenol, aromatic and enzyme. Among the four big groups, the phenol group is used more in pharmacy, body and beauty treatment. Polyphenol contained in green tea serves to inhibit the work of tyrosinase enzyme serving in melanin synthesis. Including in phenol are flavanol and catechin. Catechin, particularly EGCG, is one compound existing in green tea and has antioxidant activity. Other proof shows that green tea catechin is able to neutralize free radicals 100 times more effectively than vitamin C and 25 times more potently than vitamin E (Hussain et al., 2016). Green tea in extract form has been used to protect skin from ultra violet rays hazard (Lim et al., 2014). Considering its antioxidant nature and role in inhibiting tyrosinase activity, it is much used in beauty, health sectors and as skin whitener (Woolery-Lloyd & Kammer, 2011). Referring to the data, a hypothesis may be made that green tea extract has more potent antioxidant activity than tocopherol. Therefore, the research purpose is to evaluate whether applying green tea extract cream is more effective than tocopherol to reduce tyrosinase level and the amount of melanin in rats exposed to UVB.

METHODS

This experimental research apply a Post Test Only Control Group design. Thirty male wistar rats (Rattus novergicus), 2 months old, were divided into 3 groups. The control group (C-G) was smeared with basic cream; Green tea group (GT-G) was smeared with green tea cream; Tocopherol group (TC-G) was smeared with tocopherol cream. Before commencement, all rats were acclimatized by group for one week. Before treatment, all rats' back was shaved, exposed to UVB rays and smeared with cream according to their respective group for 0.2 mg/cm2 of rat skin surface area. UVB rays exposure was performed to all rats every Monday, Wednesday and Friday for 4 weeks, while the cream was smeared every day. The creams were smeared on the same day with UVB exposure 20 minutes before exposure and 4 hours after exposure. The dose of UVB exposure was 50 mJ/cm2 for 50 seconds in the first week, 70 mJ/cm2 for 70 seconds in the second week and 80 J/cm2 for 80 seconds in the next two weeks, thus the total UVB exposure for 4 weeks was 840mJ/cm2 (Miot, Brianezi, Tamega, & Miot, 2012). The rats were left for twenty four hours after the last radiation in order to remove the effect of acute radiation. In the end of research, blood was taken from orbital sinus to

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count the amount of melanin and tyrosinase level. The amount of melanin was measured with pixel method and tyrosinase level was measured with ELISA method. This research was conducted upon approval from the ethics committee of Faculty of Medicine, Unissula no.92/II/2018/Komisi Bioetik.

Basic Cream Preparation

Stearic acid 14.5gram was inserted into porcelain dish, put on boiled water and stirred until melted. Borax 125mgwas added and stirred, then tri ethanolamine 1.5ml was inserted and stirred. Further, glycerin 10ml was inserted and stirred, added with distilled water 25ml bit by bit until homogenously mixed and cream base was formed.

Green Tea Cream Preparation

Ingredient preparation undergo leaf weathering, cooling, grinding, drying and sorting phases. The unprocessed natural ingredient preparation was then extracted by dissolving it in ethanol 60%. It was then extracted with M AEdaya microwave 450W for 8

Table 1. Data of Mean Amount of Melanin and Tyrosinase Level

Variable	Groups			p-value
	C-G N=10 Mean±SD	GT-G N=10 Mean±SD	TC-G N=10 Mean±SD	
Melanin (%)	17.81(±1.93)	5.97(±1.59)	9.82(±1.72)	>0.05* >0.05** <0.05***
Tyrosinase (ng/ml)	2.23(±0.49)	1.023(±0.78)	2.05(±0.71)	>0.05* >0.05** <0.05***

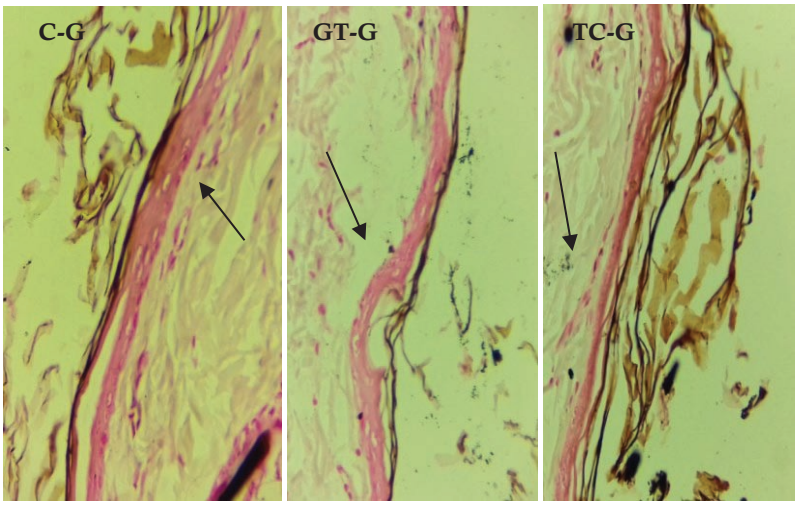

Figure 1. Amount of melanin obtained from Masson-FontanaDying methods on Skin Epidermis in each group

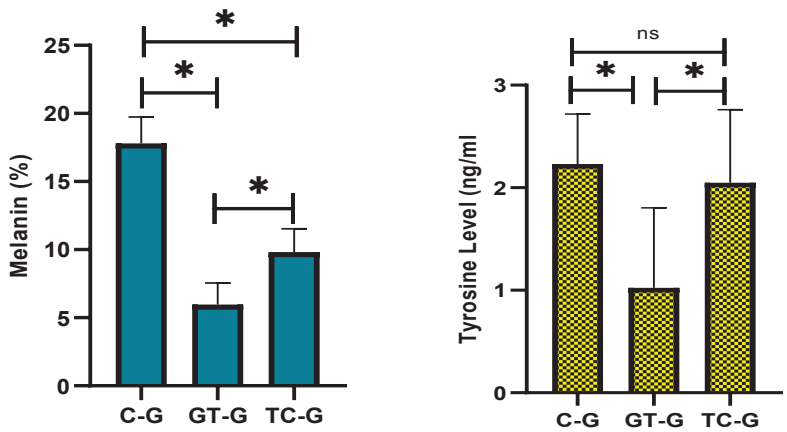

Figure 2. Post Hoc statistical analyis on Melanin and Tyrosinase between group. * p<0.05; ns: not significant

minutes.

Tocopherol Cream Preparation

This research uses lotion of pharmaceutical product from a factory containing tocopherol obtained from pharmacy.

Tyrosinase Level Examination

Ophthalmic vein blood preparation was taken from each rat for 1ml and anti-coagulation EDTA-Na2 was applied. The blood was centrifuged for 15 minutes at the speed of 1000 rotations at 2-80C. The supernatant produced was immediately tested after the total volume of working solution x number of wells to be used was prepared. The examination method used was ELISA.

Melanin Amount Measurement

Biopsy on rat skin area of 2 x 2cm was made, then immersed into 40% formalin solution. Preparation was made through fixation, dehydration, clearing, embedding and cutting phases, then dying with Masson-Fontana. The dying results are in the form of black melanin granule with pink cell nucleus and pale pink cytoplasm. Counting was then performed using digital analysis method, where each preparation was shot using camera Optilab Pro and microscope Olympus CX21FSI at 400x magnification. The field of view was where melanin exists the most as marked with black area. The counting was performed with the formula Amount of melanin= (Melanin pixel)/(Epidemis pixel) ×100%.

Data Analysis

The data collected were then analyzed using Shapiro-Wilk test to determine the data distribution. The analysis result shows that the data were normally distributed, $p>0.05$. Homogeneity test was then conducted with Levene's test. The analysis result shows that the data were homogenous, $p>0.05$. Considering that the data were normally distributed and homogenous, a One Way Anova test was conducted, followed with Post Hoc LSD test. The analysis result is significant if $p<0.05$.

RESULTS

After application of topical cream for 4 weeks, results were obtained as presented in table 1.

The research results show that the lowest amount of melanin is that of group GT-G, followed with group TC-G and the highest is of the control group C-G (figure 1). Similar with the tyrosinase level, group GT-G has the lowest tyrosinase level, followed with group TC-G, and the highest level is that of control group (C-G). A statistical analysis needs to be conducted to examine whether there is difference in the amount of melanin and tyrosinase level between the groups. Considering that the data is normally distributed and homogenous, the statistical test used is Anova. The result of Anova shows that there is significant difference between the amount of melanin and tyrosinase level of the three groups, $p<0.05$. Further, a Post Hoc LSD test needs to be conducted to examine which group is significantly different, as explained below.

Amount of Melanin

The amount of melanin of groups GT-G and TC-G is significantly lower than that of group C-G, $p<0.05$. The amount of melanin of group GT-G is significantly lower than that of group TC-G, $p<0.05$ (figure 2). This describes that green tea's effect of reducing melanin amount is better than that of tocopherol.

Tyrosinase Level

The level of tyrosinase of group GT-G is significantly lower than that of groups C-G and TC-G, $p<0.05$. The level of tyrosinase of group TC-G is insignificantly lower than that of group C-G, $p>0.05$ (figure 2). This describes that green tea's effect of reducing tyrosinase enzyme level is better than that of tocopherol.

DISCUSSION

This research result shows that green tea cream application may reduce melanin amount and tyrosinase level. Meanwhile, tocopherol cream, even if it may reduce melanin amount, but cannot reduce tyrosinase level. This describes that the effect of green tea cream application is better than tocopherol cream application. This research result is in line with the invitro study conducted by Kim CY, et al. on melanin cell A, stating that green tea application evidently reduces tyrosinase enzyme activity (Young Chul Kim et al., 2015). Other invitro study reported by Dissanayake CY, et al. also states that green tea application to B16-F10 melanoma cell evidently inhibits melanin synthesis through reduction of gene expression responsible for melanin synthesis (Dissanayake, Moon, Yang, Lee, & Han, 2018). On the other hand, tocopherol cream application, even if it may reduce melanin amount, but it cannot reduce tyrosinase level significantly. This research result is slightly different from the in vitro study on B16 melanoma cell, stating that various analog tocopherols evidently reduce melanin amount and

tyrosinase activity significantly (Albakry, Amiyati, & Hasan, 2018). Among the 8 analog tocopherols, that ones with high potential are D-β-tocopherol and D-γ tocopherol (Suárez-Jiménez et al., 2016). In this study, the analog tocopherol used is D-α.

Tocopherol cream, even if there is reduction of tyrosinase level, but it is insignificant compared to the control. Other study conducted by Keen & Hassan (2016) also shows that α-tochopherol only has minimal depigmentation effect on melasma, through lipid peroxidation on melanocyte membrane, intracellular glutathione binding and tyrosinase inhibition (Keen & Hassan, 2016).

The insignificant reduction of tyrosinase level is expectedly also related to gene polymorphism. The study conducted by Tripathi et al. states that general non-pathologic polymorphism is identified in tyrosinase gene of Caucasian race, with codon 402 in the form of CGA (arginine) or CAA (glutamine) (Tripathi, Giebel, Strunk, & Spritz, 2018). In addition, melanin synthesis is also influenced by tyrosinase related Protein 1 (TYRP1) and Dopachrome T automerase (DCT). The three enzymes works together and synthesize two types of melanin, namely eumelanin and pheomelanin (Videira et al., 2013). Polymorphism also occurs to black American people, but codon 402 CAA is not detected in Oriental population. Tyrosinase activity with codon 402 Gln is also influenced by temperature, in which activity is reduced 75% at 37°C compared to activity at 31oC, while the enzymatic activity of codon 402 Arg is not influenced by temperature (Cichorek, Wachulska, Stasiewicz, & Tyminska, 2013; D'Orazio, Jarrett, Amaro-Ortiz, & Scott, 2013).

The reduction of melanin amount because of green tea application is caused by flavonoid's antioxidant nature and able to inhibit tyrosinase enzyme activity (Woolery-Lloyd & Kammer, 2011; Young Chul Kim et al., 2015). The most important flavonoid existing in green tea is Epigallocatechin Gallat (EGCG). EGCG is able to inhibit tyrosinase activity and prevent tyrosinase formation. Thus, leading to melanin synthesis inhibition. EGCG as natural antioxidant will catch free radicals arising from UVB rays exposure producing Reactive Oxygen Species (ROS) (Peluso & Serafini, 2017). ROS will influence mRNA tyrosinase regulation, triggering formation of tyrosinase, which catalyzes the main reaction of melanogenesis by hydroxylation L-tyrosine to L-dopa, then oxidizing L-dopa to dopaquinone. Melanin synthesis produces eumelanin after passing through indol quinon pathway or become pheomelanin after passing through cysteinyl DOPA pathway (D'Orazio et al., 2013).

This melanin amount reduction conforms to previous studies that green tea's polyphenol serves as photoprotective agent and able to prevent skin pigmentation due to UVB rays exposure. This depigmentation occurs since green tea contains abundant catechin in the form of natural antioxidant (Oliveira, 2012). The research conducted by Chasissa M, et al. (2016) also states that flavonoid is natural polyphenol able to depigment skin by directly inhibiting tyrosinase activity (Charissa, Djajadisastra, & Elya, 2016). In addition, tannin is strong antioxidant and able to inhibit tyrosinase (Chai et al., 2014). Further, various studies also state that antioxidant may suppress oxidant's negative effect, bind free radicals and inhibit melanin synthesis (Nichols & Katiyar, 2011).

On the other hand, some studies also state that tocopherol may directly inhibit tyrosinase activity in melanogenesis mechanism(Suárez-Jiménez et al., 2016) and even reduce up to 70% pigmentation and reduce 40% tyrosinase activity compared to the control group (Keen & Hassan, 2016). Therefore, we may state that green tea application may inhibit melanin formation. Similarly, tocopherol may inhibit melanin formation, but in this research, green tea does it more significantly.

This study's limitation is that it does not examine the level of antioxidant, pharmacokinetics and pharmacodynamics of green tea or tocopherol influencing melanin amount and tyrosinase level.

CONCLUSION

The research results show that green tea cream and tocopherol cream applications are able to reduce tyrosinase activity and melanin synthesis. However, green tea's capability to reduce melanin amount and tyrosinase level is better than tocopherol.

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CONFLICT OF INTEREST

There is no conflict of interest in this publication.

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